The first complete mitogenome of *Cyclommatus* stag beetles (Coleoptera: Lucanidae) with the phylogenetic implications

Jing LIU[1], Chenggong LI[2], Shuo YOU[1], Xia WAN[1①]

1. *Department of Ecology*, *School of Resources and Engineering, Anhui University, Hefei*, *Anhui 230000, China*

2. *Yunnan Provincial Key Laboratory of Entomological Biopharmaceutical*, *Dali University*, *Dali*, *Yunnan 671000*, *China*

Abstract: The first complete mitogenome of *Cyclommatus* stag beetles, *Cyclommatus vitalisi* (Coleoptera: Lucanidae) is sequenced using the next generation sequening. The genomic structure is a closed circular molecule with 17,853bp in length, comprising 13 protein-coding genes, 22 transfer RNA genes (tRNAs), 2 ribosomal RNAs (rRNAs) and a control region. The sequence has neither a gene rearrangement nor a non-coding region. The nucleotide composition is A (36. 31%), C (21. 48%), T (31. 20%) and G (11. 01%), with overall AT content of 73. 61%. The phylogenetic analysis of 13 stag beetles and another three scarab beetles show that *Cyclommatus vitalisi* shares a close ancestry with *Lucanus mazama* and *Lucanus fortunei*.

Key words: *Cyclommatus vitalisi*; mitogenome; phylogenomic analysis

无皱环锹的线粒体全基因组及其系统发育关系研究（鞘翅目：锹甲科）

刘静[1]，李成功[2]，游硕[1]，万霞[1①]

1.安徽大学资源与环境工程学院，生态系，安徽 合肥 230000；2. 大理大学，云南省生物医药研发重点实验室，云南 大理 671000

摘要：无皱环锹的线粒体全基因组采用二代测序方法第一次测得，是一个长度为 17, 853bp 的封闭式圆环。序列由 13 个蛋白质编码区，22 个 tRNA，2 个 rRNA 和一个控制区组成。没有发现基因重排现象和非编码区。线粒体全基因组的核苷酸组成为 A (36. 31%)，C (21. 48%)，T (31. 20%)，G (11. 01%)，AT 含量为 73.61%。基于 13 种锹甲和 3 种金龟子的 13 个蛋白质编码基因的系统发育分析表明无皱环锹和 *Lucanus mazama*、福运锹甲具有共同祖先。

关键词：无皱环锹；线粒体基因组；系统发育分析

Introduction

Cyclommatus vitalisi is an attractive stag beetle due to the vividly yellow brown colour, strongly shiny body, smoothly large head, impressively curved male mandibles (Fig. 1). Like most members in Lucanidae, this species has distinct sexual dimorphism and male polymorphism. The males have enlarged mandibles and are much larger than the females.

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① Corresponding author, E-mail: wanxia@ahu.edu.cn

Within the males, the mandibles, head and pronotum present fascinatingly allometric variations. Currently, it has been popular pet and valuable specimen collection due to its marvelous appearance. However, this species is not common because of its narrow distribution, which merely can be found in the boundary region from Southeast Yunnan, China to Northern Vietnam (Pouillaude 1913; Didier 1925; Lacroix 1988; Mizunuma & Nagai 1994; Krajcik 2001, 2003; Fujita 2010). According to the field investigation, we would say that the future of *C. vitalisi* is not so optimistic as a result of the excessive hunting, habitat fragmentation and the climate anomalies. Although we have not yet evaluated any necessity for its conservation in China. In other areas, some stag beetles, such as *Lucanus cervus*, *L. datunensis*, and *Neolucanus swinhoei*, have been treated as conservation species. The most famous case is that all of the 14 species in the genus *Colophon* have been listed in the IUCN red list. (http: //www.iucnredlist.org). Molecular data have helped to deeply understand the evolution of these stag beetles (Cox *et al*. 2013; Lin *et al*. 2011; Tsai *et al*. 2014; Switala *et al*. 2014).

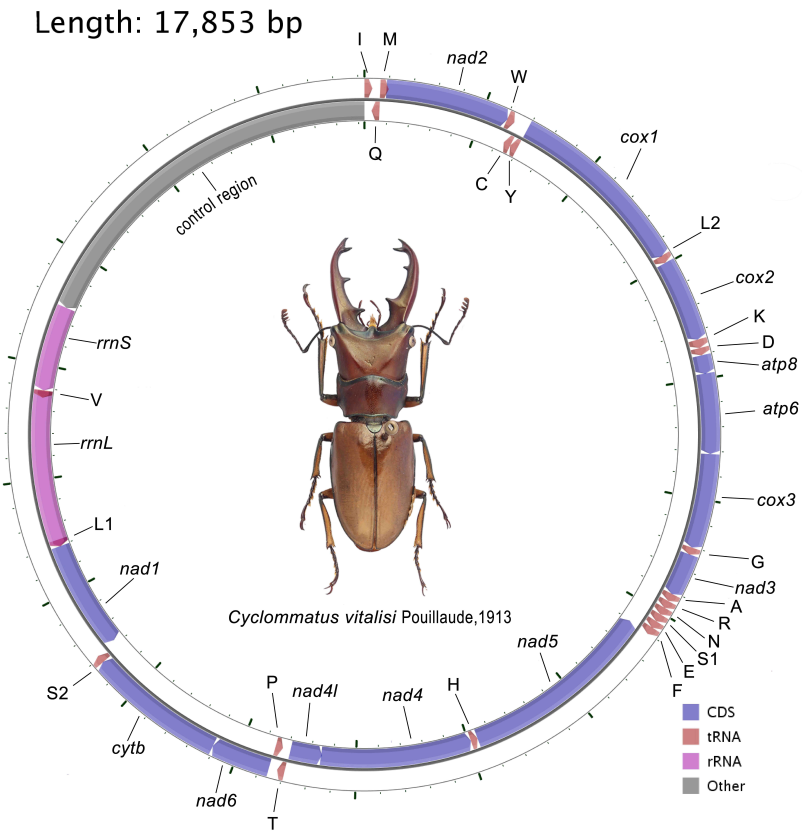

Figure 1. The closed circular molecule of the *C. vitalisi* mitogenome.

The mitogenome could provide informative data for the studies of conservational genetics, molecular evolution and phylogenetic analysis in beetles and other insects (Simon *et al*. 2006; Cameron 2014; Crampton-Platt *et al*. 2015; Timmermans *et al*. 2016; Yuan *et al*. 2016). However, the mitogenomic study in Lucanidae still in its early days. So far, only five complete mitogenomes of lucanid beetles have been reported belonging to three genera *Lucanus*,

Prosocopoilus and *Sinodreon* (Sheffield *et al*. 2009; Kim *et al*. 2013; Wu *et al*. 2016; Lin *et al*. 2017). There is lack of the mitogenomic data of the popular *Cyclommatus* stag beetle. In this study, we sequenced the complete mitogenome of *C*. *vitalisi* for the first time using the next generation method. And we hope that this genomic data could be useful for its future conservational work and a comprehensive phylogenetic analysis of Lucanidae.

Material and methods

Sample Collection and DNA Extraction

The voucher specimen of *C*. *vitalisi* was collected from Mt. Huanglianshan, Lvchun County, Yunnan Province in August, 2015 by Dr. Zizhong YANG. Total genomic DNA was extracted from the muscle of a single *C*. *vitalisi* using the Qiagen DNAeasy Kit. The specimen deposited in the Museum of Anhui University and the sequence was submitted to GenBank and assigned accession number MF037205.

Polymerase Chain Reaction Amplification, and Sequencing

PCR amplification reactions were carried out in 25 μL volumes containing 10 μM of each primer (forward and reverse) 1 μL, 2 μL template DNA, 12.5 μL 2×EasyTaq SuperMix (+dye), and 8.5 l μL sterile double-distilled water to make up a final volume of 25μL. The polymerase chain reaction amplifications were performed under the following conditions: an initial denaturation at 94°C for 2 min, followed by 35–37 cycles of denaturation at 94°C for 40 seconds, annealing at 52–58°C for 50 seconds, and elongation at 70°C for 1 min, and then a final extension step at 72°C for 7 min. The temperature of annealing was determined by the length of fragments. Sequencing was conducted with the Illumina HiSeq 2000 platform. Cluster strands created by bridge amplification were primed and all four fluorescently labeled, and 3-OH blocked nucleotides were added to the flow cell with DNA polymerase. The cluster strands were extended in single nucleotides. Following the incorporation step, the unused nucleotides and DNA polymerase molecules were washed away, a scan buffer added to the flow cell, then the optics system scanned each lane of the flow cell in imaging units (tiles). Once imaging was completed, chemicals that effect cleavage of the fluorescent labels and the 3-OH blocking groups were added to the flow cell, which prepares the cluster strands for another round of fluorescent nucleotide incorporation.

Mitogenome Assembly, Annotation and Analysis

The mitogenomes were assembled using SOAP denovo (BGI Company, Shenzhen, China) and preliminary annotations made with the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py). tRNA genes and their secondary structures were inferred using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/). Those not identified by tRNAscan-SE, in addition to 16S ribosomal RNA (*rrnL*, *lrRNA*), and 12S ribosomal RNA (*rrnS*, *srRNA*), were determined according to sequence similarity with related species. The protein-coding genes (PCGs) were determined by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) under the invertebrate mitochondrial genetic code. Nucleotide compositions, codon usage, and relative synonymous codon usage (RSCU) values of PCGs were calculated with MEGA version 6.05 (Tamura *et al*. 2013). PCGs were translated with DNAMAN v7.0.2.176 (Lynnon Biosoft, Vaudreuil-Dorion, Canada). Mitogenomes were mapped with CGView (Grant and Stothard 2008). Composition skew analysis was conducted according to formulas AT skew =

[A - T] / [A + T] and GC skew = [G - C]/ [G + C] (Perna and Kocher 1995).

Phylogenetic Analyses

In this study, we retrieved 15 mitogenome sequences from GenBank, to this data set, we added a newly sequenced *C. vitalisi* generating a dataset of 16 taxa, each PCGs was aligned individually based on codon-based multiple alignments using the MEGA 7.0.26. Models of nucleotide substitution were selected according to the Akaike Information Criterion (AIC) with jModelTest v2.1.4 (Posada, 2008). Phylogenetic trees were generated from ML analysis using RAxML (Gillett *et al*. 2014) and Bayesian inference (BI) with MrBayes v3.2.5 (Huelsenbeck and Ronquist, 2013), both under the GTR + I + G model. Node support in the ML tree was estimated through bootstrap analysis with 1,000 replicates. The BI was conducted with two simultaneous Markov chain Monte Carlo runs of 2 million generations, sampled every 1,000 steps, with the first 25% discarded as burn-in. Phylogenetic trees were viewed and edited in Figtree v1.4.3. (Rambaut 2016).

Results and Discussion

The mitogenome of *C. vitalisi* is a closed circular molecule with 17,853bp in length (Fig. 1), with the typical gene content as other known Coleoptera mitogenomes: 22 transfer RNA genes (tRNAs), 2 ribosomal RNA genes (*rrnL* and *rrnS*), 13 protein-coding genes (PCGs) and 1 non-coding AT-rich region (Wolstenholme 1992). The base compositions of the entire major strand have a total AT content of 73. 61%. AT- and GC-skews were calculated as 0. 064 and –0. 271. All PCGs are AT-biased (79.18% on average), with the highest A+T content in *ND4L* (78.85%) and the lowest in *COI* (66.08%). As a classical invertebrate mitochondrial genome, twelve PCGs of the sequence employ initiation codons ATN, except the start codon of *COI* is AAT. The stop codons of 8 PCGs are TAA or TAG, while others use TA residue or a single T as the terminator codons. Among all tRNA genes, only the cloverleaf secondary structure of *tRNA-Ser* (AGN) is absent due to the deficiency of the dihydrouridine (DHU) arm, which is a typical feature of metazoan mitogenomes (Cameron 2014). The control region is 3,321bp long and is located between *srRNA* and *tRNA-Ile*. There is no marked non-coding region, which is similar to the known mitogenomes of *P. gracilis* and *S. yunnanense*. Whereas the two species *P. confucius* and *P. blanchardi* possess a notable non-coding region aside from the control region (Kim *et al*. 2015; Lin *et al*. 2017). Additionally, there is no gene rearrangement in *C. vitalisi*, which has only occurred in the species of *S. yunnanense* (Lin *et al*. 2017) until now. We also analyzed the phylogenetic relationships among 13 stag beetles as the in-group and another three scarabs as an out-group inferred from the sequences of 13 protein-coding genes. Nine of them are downloaded from the GenBank. Models of nucleotide substitution were selected according to the Akaike Information Criterion (AIC) with jModelTest (Posada 2008). Phylogenetic trees were generated from maximum likelihood analysis (RAxML; Gillett *et al*. 2014) under the GTR + G model and Bayesian inference (MrBayes; Huelsenbeck and Ronquist 2013) under the GTR + I + G model. The resolution of maximum likelihood and Bayesian analyses (Fig. 2) both indicates that *C. vitalisi* shares a close ancestry with *L. mazama* and *L. fortunei*.

In conclusion, the first complete mitogenome of genus *Cyclommatus*, *C. vitalisi* is a typical metazoan mitogenome, and the sequence has neither gene rearrangement as in *S. yunnanense* nor a non-coding region as in *P. confucius* and *P. gracilis*.

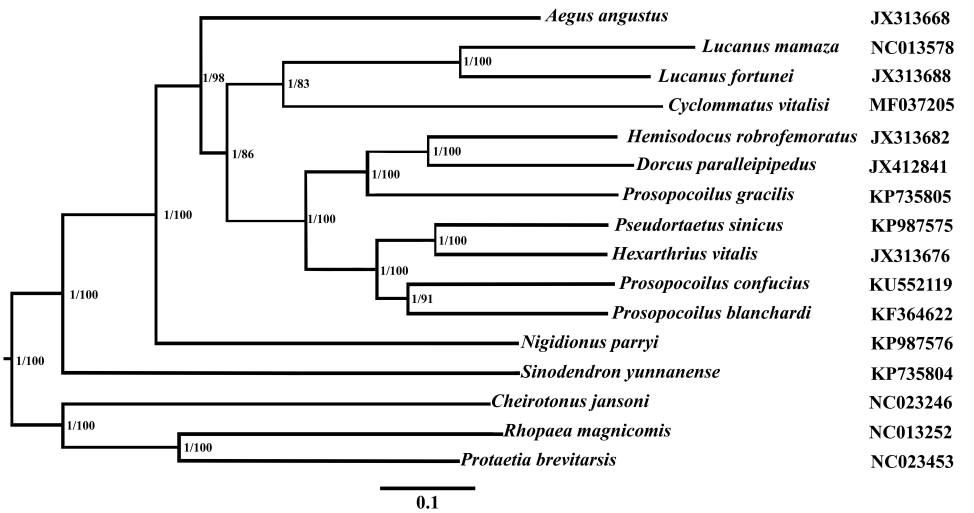

Figure 2. The ML and BI phylogenetic trees of *C. vitalisi* and 15 other species based on 13 PCGs. GTR+G and GTR + I + G were selected as the best models, and with three scarab species as outgroups.

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